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# Characterization of the Reverse Transcriptase of a Human Immunodeficiency Virus Type 1 Group O Isolate

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The catalytic properties and sensitivity to different inhibitors have been determined for the reverse transcriptase (RT) of group O human immunodeficiency virus type 1 (HIV-1). The RT-coding region was cloned from a new HIV-1 group O isolate from Spain, expressed in *Escherichia coli,* and purified by affinity chromatography. This new RT showed 79% amino acid sequence identity with the corresponding enzyme of group M subtype B strain BH10. The two enzymes showed very similar kinetics of RNA-dependent DNA polymerization using homopolymeric template-primers and RNase H specific activity. Inhibitor sensitivity to ddTTP and 3′-azido-2′,3′-dideoxythymidine triphosphate (AZTTP) was also similar for both enzymes. However, the two enzymes differed dramatically in their sensitivity to several inhibitors. While the RT of the BH10 isolate was sensitive to nevirapine and loviride ($IC_{50}$ ranged from 0.16 to 8.2 $\mu M$, depending on the substrates used), the enzyme of the Spanish HIV-1 group O isolate showed high-level resistance to those compounds ($IC_{50} > 200$ $\mu M$). The amino acid sequence of the RT of group O HIV-1 contains three amino acids (Cys-181, Glu-179, and Gly-98), which are found in group M subtype B strains resistant to nonnucleoside RT inhibitors. The recombinant group O HIV-1 RT should be useful for studies aimed at discovering and designing drugs directed toward group O isolates of HIV-1. 

## INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) displays a high degree of genetic variability *in vivo.* Two major groups have been defined to classify the circulating HIV-1 strains: Group M (main) and Group O (outlier). Group M can be further subdivided into at least 10 different subtypes (A–J), which are widely distributed around the world. Phylogenetic analysis of the *env* region of these subtypes shows that they are approximately equidistant from one another, producing a "star" phylogeny. The first HIV-1 of group O (ANT70) was isolated in 1987, from two Cameroonians living in Belgium, and a partial nucleotide sequence of the virus was reported 3 years later (De Leys *et al.,* 1990). Recent epidemiological data revealed that HIV-1 group O infection is widely distributed in Cameroon and probably in the neighboring countries Gabon and Nigeria (Mulanga Kabeya *et al.,* 1995; Delaporte *et al.,* 1996; Mauclère *et al.,* 1997; Peeters *et al.,* 1997). Its prevalence has been estimated to be around 6 to 8% among HIV-infected people in urban areas of Cameroon (Gürtler *et al.,* 1994; Mauclère *et al.,* 1997). In addition, HIV-1 group O infection has been identified in other countries of West and South East Africa (Heyndrickx *et al.,* 1996; Songok *et al.,* 1996; Peeters *et al.,* 1997), as well as in Europe (Loussert-Ajaka *et al.,* 1995; Hampl *et al.,* 1995; Soriano *et al.,* 1996), and the U.S. (Gould *et al.,* 1996). In one case, HIV-1 group O has been isolated from a Caucasian woman living in France with no known ties to Africa (Charneau *et al.,* 1994). All these data suggest that this group of HIV-1 has begun to spread to many areas of the world from its likely original source in Central Africa.

Analysis of group O strains shows that they are highly divergent from other subtypes and are almost as close to HIV-2 as to HIV-1. Conventional screening tests (Western blot, ELISA, or polymerase chain reaction) designed to detect HIV-1 or HIV-2 in infected individuals can fail to detect infection by HIV-1 group O viruses (Loussert-Ajaka *et al.,* 1994; Schable *et al.,* 1994). The diversity of group O strains is most remarkable at the V3 loop region of gp120. The tip of the loop of the characterized group O strains does not contain the GPG motif, which is present in most group M isolates (Blouin *et al.,* 1996). Instead, the V3 loop of group O HIV-1 strains bears the motifs GPM or GPL (De Leys *et al.,* 1990; Charneau *et al.,* 1994; Gürtler *et al.,* 1994; Loussert-Ajaka *et al.,* 1995; Korber *et al.,* 1996; Mas *et al.,* 1996; Mauclère *et al.,* 1997). Unlike HIV-1 group M strains, group O strains as well as other primate immunodeficiency viruses do not require cyclophilin A for replication (Braaten *et al.,* 1996). Taken together, these observations suggest that both HIV-1 groups were transmitted to humans from other primate species in two separate events.

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HIV-1 group O infection poses a potential public health risk, and successful antiviral strategies must be able to cope with the observed differences between group O and group M viruses. The reverse transcriptase is an important target for therapeutic intervention. Studies on the phenotypic sensitivity to antiretroviral drugs performed using HIV-1 group O isolates recovered from infected individuals revealed that group O viruses were sensitive to the reverse transcriptase inhibitors AZT (zidovudine), ddI (didanosine), and ddC (zalcitabine) and to the protease inhibitor saquinavir (Descamps *et al.,* 1995). However, they were resistant to the nonnucleoside reverse transcriptase inhibitors delavirdine and TIBO R82913 (Descamps *et al.,* 1995). Here, we report on the characterization of the enzymatic properties and inhibitor sensitivity of a recombinant HIV-1 group O reverse transcriptase that has been expressed in *Escherichia coli* and bears the amino acid sequence derived from an HIV-1 group O strain previously isolated in Spain (Soriano *et al.,* 1996).

## MATERIALS AND METHODS

### Viral isolates and preparation of DNA template

Peripheral blood mononuclear cells (PBMCs) from a 35-year-old man infected with group O HIV-1 (ESP1 isolate) (Soriano *et al.,* 1996) were collected at the Centro de Investigaciones Clínicas, Instituto de Salud Carlos III (Madrid, Spain) in April and September 1995. Proviral DNA was extracted directly from uncultured PBMCs as previously described (Quiñones-Mateu *et al.,* 1995).

### Polymerase chain reaction (PCR) amplification and plasmid construction

Genomic regions encoding the 66-kDa subunit of the RT were amplified using a "nested" polymerase chain reaction and specific oligonucleotide primers (Table 1). PCR amplifications were all done in 10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin, a 0.2 m*M* concentration of each of the four deoxynucleotide triphosphates, 200 ng of each primer, and 1 unit of *Taq* polymerase (Perkin–Elmer–Cetus, Norwalk, CT) in a volume of 100 $\mu$l. One microgram of template DNA and primers RTO1 and RTO2 (Table 1) were used in the first external amplification. PCR amplification was initiated with an incubation at 94° for 5 min, followed by 3 cycles of 94° for 1 min, 55° for 1 min, and 72° for 1 min; 32 cycles of 94° for 15 sec, 55° for 45 sec, and 72° for 1 min; and a final incubation at 72° for 10 min. Samples of the products of the primary reaction were reamplified using nested inner primers RTO3 and RTO4 (Table 1). This amplification was done using the same conditions as for the external PCR. The products, including negative controls (without DNA), were examined by agarose gel electrophoresis and purified using a commercially available kit (Geneclean II kit, Bio101, Vista, CA). Direct sequencing of the PCR-amplified DNA was performed using the femtomole method (Promega) followed by treatment of the reaction mixture with terminal deoxynucleotidyl transferase (Quiñones-Mateu *et al.,* 1996). Primers used in the sequencing reactions are given in Table 1.

The purified DNA obtained from the nested PCR was cleaved with *Nco*I and *Eco*RI, and cloned into the expression vector pRT6. This vector derives from p66(RT), a pUC plasmid that when introduced in *E. coli* induces the synthesis of large quantities of the 66-kDa subunit of HIV-1 RT (Hizi *et al.,* 1988). To facilitate cloning, we introduced an *Eco*RI site at the 3′ end of the RT coding region of p66(RT). For such a purpose, 20 pmol of oligonucleotides Eco35 and Eco53 (Table 1) were phosphorylated with T4 polynucleotide kinase (Boehringer) in the presence of 1 m*M* ATP. Equal amounts of each primer were then mixed, incubated at 95° for 3 min and slowly cooled at room temperature, to form a duplex with two cohesive ends. These overhangs are compatible with the cohesive ends obtained after digestion of p66(RT) with *Sal*I and *Hin*dIII. Ligations and transformations were done following standard protocols. After cloning the cleaved product of the nested PCR in plasmid pRT6, the nucleotide sequence of selected clones harboring the coding region of the 66-kDa subunit of the RT was determined and compared to the sequence obtained from the PCR product.

The coding region of the 51-kDa subunit of the HIV-1 RT was cloned in the expression vector pTrcHisB (Invitrogen Corp.). For such a purpose, a DNA fragment containing the nucleotide sequence of p51 was synthesized by PCR using primers RTO7 and RTO8 (Table 1). PCR was done as described above. A plasmid derived from pRT6 and containing the coding region of the 66-kDa subunit was used as template DNA. The amplified DNA was analyzed on an agarose gel, purified, and cleaved with *Nhe*I and *Eco*RI. Then, it was cloned into the plasmid pTrcHisB, previously digested with both enzymes.

### Protein expression and purification

Purification of recombinant RTs was carried out after independent expression of their subunits, by following a previously described procedure (Martín-Hernández *et al.,* 1996). The *E. coli* strains used in these experiments were DH5$\alpha$ and the Epicurean TOPP cells (Stratagene). As a consequence of the cloning scheme, the 66-kDa subunit contains two additional amino-terminal residues (Met-Val-) which are not found in the viral RT (Hizi *et al.,* 1988). The 51-kDa subunit was obtained with an extension of 14 amino acid residues at its N-terminal end which includes six consecutive histidines to facilitate its purification by metal chelate affinity chromatography (LeGrice *et al.,* 1991). The purity of the enzyme was assessed by sodium dodecyl sulfate–polyacrylamide gel electropho-

TABLE 1

Synthetic Oligonucleotides Used as Primers for Polymerase Chain Reaction (PCR), Nucleotide Sequencing, and Plasmid Modification

| Oligonucleotide | Sequence (5′ → 3′) | Orientation | Position[a] |
|---|---|---|---|
| RTO1 | GGAACAGTATTGGTGGGACC | Sense | 2524 |
| RTO2 | TTCACTAGCTAGTGCTTTCC | Antisense | 4340 |
| RTO3 | GTACACCCATGGTCCCTATAAGCCCCATAGCCC | Sense | 2591 |
| RTO4 | GGTCTATTCGAATTCATCACAGGACTCTTCTAATATC | Antisense | 4267 |
| RTO5 | TAGGTATCCCACATCCGGGG | Sense | 2879 |
| RTO6 | TCTTTGGAATCCTGTAAGGC | Antisense | 4057 |
| RTO7 | GGAAGGATCCGCTAGCCCTATAAGCCCCATAGCC | Sense | 2605[b] |
| RTO8 | GCTCCATGAATTCAATAGGTTTCTGCCCCC | Antisense | 3909 |
| RTO9 | ATCAGTGGGGATTCACTACC | Sense | 3233 |
| RTO10 | ATTCCTGAGACACCTTCTGG | Antisense | 3720 |
| Eco35 | AGCTTGGGCTGCAGAATTCG | — | — |
| Eco53 | TCGACGAATTCTGCAGCCCA | — | — |

[a] The number given corresponds to the position of the 5′ terminus of the HIV-1 ANT70 sequence as shown under GenBank Accession No. L20587 (Vanden Haesevelde *et al.,* 1994).

[b] The first 15 bases of this oligonucleotide are complementary to the DNA sequence of plasmid pRT6. Position number refers to the underlined nucleotide.

resis. RT concentrations were determined using the Bio-Rad protein assay.

## Enzymatic assays

DNA polymerase activity assays were carried out in 50 m*M* Tris–HCl (pH 8.0), 20 m*M* NaCl, 10 m*M* $MgCl_2$, 8 m*M* dithiothreitol, 3–5 $\mu$Ci/ml [$^3$H]dTTP or [8-$^3$H]dGTP, and 0.5–1 $\mu M$ template-primer (concentration expressed as 3′-hydroxyl primer termini). The template-primers used were poly(rA)·oligo(dT)$_{20}$, poly(rA)·oligo(dT)$_{12-18}$, poly(rC)·oligo(dG)$_{12-18}$, and poly(dC)·oligo(dG)$_{12-18}$. Poly(rA)·oligo(dT)$_{20}$ was prepared by mixing 52 $\mu$l of 100 $\mu M$ phosphorylated oligo(dT)$_{20}$ (Pharmacia), 6 $\mu$l of poly(rA)$_{\sim 450}$ (Pharmacia) at 700 $A_{260}$ units/ml, and 40 $\mu$l of distilled water. Poly(rA)·oligo(dT)$_{12-18}$ and poly(rC)·oligo(dG)$_{12-18}$ (Pharmacia) were dissolved in distilled water at 14.0 and 16.0 $A_{260}$ units/ml, to obtain stock solutions with a primer concentration of 100 $\mu M$. Poly(dC)·oligo(dG)$_{12-18}$ was prepared by mixing 25.4 $\mu$l of phosphorylated oligo(dG)$_{12-18}$ (400 $\mu M$) and 86 $\mu$l of poly(dC)$_{\sim 420}$ (Pharmacia) at 29.3 $A_{260}$ units/ml. In all cases, the template–primer mixtures were annealed at 90° for 5 min and slowly cooled at room temperature prior to use. For the determination of kinetic parameters, the dTTP concentration was adjusted with nonradioactive nucleotide and ranged from 1 $\mu M$ to 500 $\mu M$. Reactions (30 $\mu$l) were initiated by the addition of 0.8–3 pmol of enzyme, incubated at 37° for 10–30 min, and terminated by adding 20 $\mu$l of 0.5 *M* EDTA. The amount of polymerized deoxynucleotide was determined by acid insoluble precipitation (Martín-Hernández *et al.,* 1997). Kinetic parameters of polymerization were calculated by fitting the data to the Michaelis–Menten equation as previously described (Martín-Hernández *et al.,* 1996).

The dissociation constants ($K_d$) for poly(rA)·oligo(dT)$_{20}$ binding to HIV-1 RT were determined as the apparent $K_m$ values for template-primer binding, when the concentration of dTTP was held fixed at $\frac{1}{5}$ of its $K_m$ value, determined at saturating concentrations of template-primer (Reardon *et al.,* 1991).

RT inhibition was studied using ddTTP (Pharmacia), AZTTP (Moravek Biochemicals, Brea, CA), nevirapine, and loviride ($\alpha$-anilinophenylacetamide derivative R 89439). Nevirapine and loviride were kindly provided to one of us (V.S.) by Boehringer Ingelheim Pharmaceuticals and The Wellcome Foundation Ltd., respectively. The $K_i$ values for ddTTP and AZTTP were determined in the presence of saturating concentrations of template-primer, by using two different methods. First, the inhibition of the DNA polymerase activity of the RT was measured by varying the concentration of ddTTP or AZTTP, in the presenced of a fixed concentration of dNTP, as previously described (Fan *et al.,* 1996). Then, apparent $K_m$ values measured in the presence of fixed concentrations of inhibitor (at around 1–4 times the $K_i$ obtained using the previous method) were determined by fitting the velocity data to the Michaelis–Menten equation as above. Inhibition constants were calculated from the equation $K_{m(\text{app})} = K_m\,(1 + [\text{I}]/K_i)$, derived for competitive inhibition. In the experiments carried out with nevirapine and loviride, the $IC_{50}$ values were determined with respect to [$^3$H]dTTP or [8-$^3$H]dGTP incorporation. The corresponding template-primers and dNTPs were used at 1 and 2 $\mu M$ concentration, respectively.

RNase H activity assays were performed in the presence of $MgCl_2$, using [$^3$H]Poly(rA)·Poly(dT) as substrate, as previously described (Martín-Hernández *et al.,* 1997). RNase H activity is expressed in units, where one unit is defined as 1 nmol of trichloroacetic acid-soluble radiolabeled adenylate released in 1 hr at 37°.

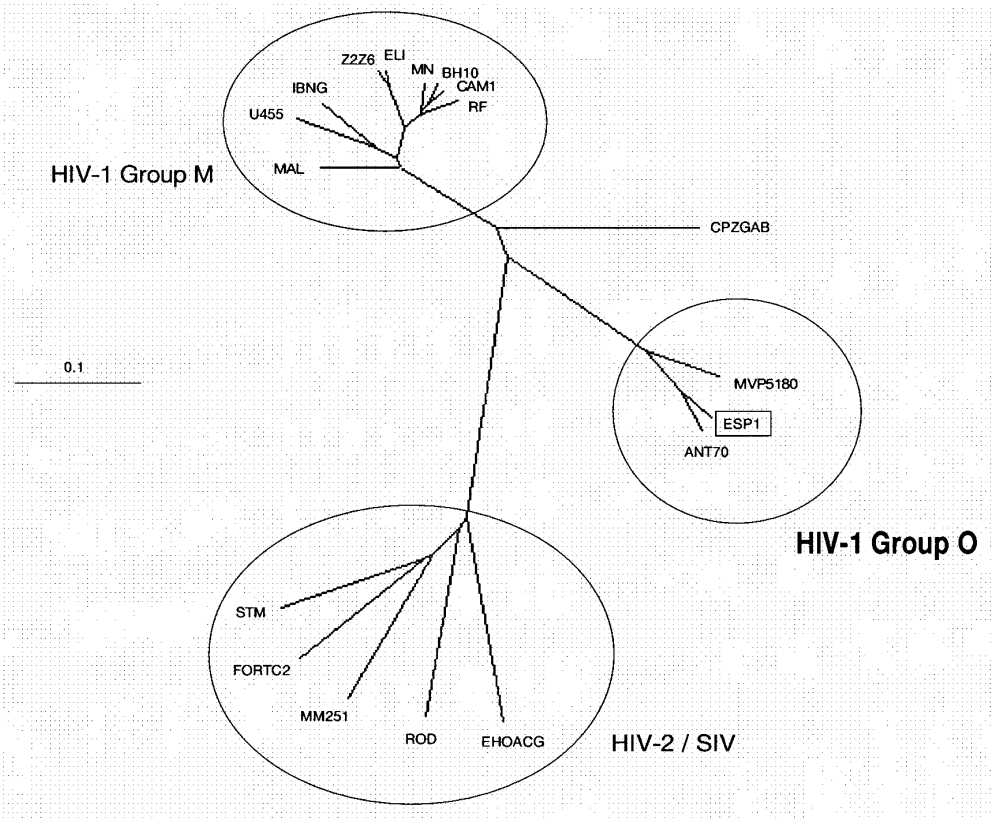


FIG. 1. Phylogenetic tree analysis of the RT-coding region of an HIV-1 group O nucleotide sequence from Spain (boxed), with HIV-1, HIV-2, and simian immunodeficiency virus (SIV) isolates from the current database at Los Alamos National Laboratory. Group M and group O isolates are indicated. GenBank accession numbers for reference sequences are as follows: U455, M62320; IBNG, L39106; MN, M17449; CAM1, D10112; RF, M17451; BH10, M15654; ELI, K03454; Z2Z6, M22639; MAL, K03456; ANT70, L20587; MVP5180, L20571; ESP1, U97171; ROD, M15390; EHOACG, U27200; FORTC2, M87111; CPZGAB, X52154; MM251, M19499; and STM, M83293. Neighbor-joining consensus trees were constructed using the NEIGHBOR, SEQBOOT, and CONSENSE programs of the PHYLIP package (Felsenstein, 1989). The lengths of the branches are proportional to the relative evolutionary distances.

## RESULTS

### Nucleotide and deduced amino acid sequence of the Spanish HIV-1 group O isolate

The average nucleotide sequence of the RT coding region of the Spanish isolate was compared with others obtained from representative HIV-1 strains available from the current database at Los Alamos National Laboratory. A phylogenetic tree derived from this comparison is shown in Fig. 1. The RT *pol* sequence of the Spanish isolate ESP1 clusters within HIV-1 group O isolates and is closer to the ANT70 strain. The group O strains ANT70 and MVP5180 were isolated from symptomatic patients from Cameroon. Their reported nucleotide sequences were obtained from proviral DNA prepared from persistently infected cell cultures (Vanden Haesevelde *et al.,* 1994; Gürtler *et al.,* 1994). The cell lines used were MT4 in the case of ANT70 and HUT-78 in the case of MVP5180. The sequence of the ESP1 isolate derives from the direct amplification of proviral DNA found in the uncultured PBMCs of an infected individual. The genetic distances between the RT coding region of ESP1 and its equivalent regions in ANT70 and MVP5180 were around 5 and 10%, respectively, and were similar to those found between isolates from different subtypes of group M HIV-1 (Table 2). In contrast, the values obtained in comparisons between group O and group M isolates ranged from 31 to 34%. The primary structure derived from the RT sequence of ESP1 is given in Fig. 2, together with those obtained from other HIV-1 group O isolates and BH10 strain. HIV-$1_{BH10}$ is a representative clone of subtype B, which is the prevalent subtype in Europe, Japan, and the U.S. Pairwise comparisons between the RTs of group O HIV-1 isolates revealed that the number of amino acid differences between them ranged from 5 to 8%. This value raised to 21% when the consensus sequence for group O viruses was compared with the sequence of the BH10 isolate. Amino acid changes appeared to be scattered along the sequence, with a higher number of differences in the RNase H domain than in the DNA polymerase domain of the RT (28% versus 15%, in comparisons between BH10 and the consensus O sequence). However, relevant differences at the DNA polymerase domain of the RT were detected in positions

TABLE 2

Pairwise DNA Distance Matrices[a] among the Group O HIV-1 *pol* Sequence from Spain and Representative Sequences of Different Groups and Subtypes of HIV-1, HIV-2, and Simian Immunodeficiency Viruses (SIV)

| | HIV-1 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Group M | | | | Group O | | HIV-2 | SIV | |
| | U455 (Subtype A) | BH10 (Subtype B) | ELI (Subtype D) | MAL (subtype U)[b] | MVP5180 | ANT70 | ROD (Subtype A) | CPZGAB | MM251 |
| U455 | — | — | — | — | — | — | — | 0.27 | 0.52 |
| BH10 | 0.12 | — | — | — | — | — | — | 0.27 | 0.50 |
| ELI | 0.13 | 0.07 | — | — | — | — | — | 0.27 | 0.50 |
| MAL | 0.12 | 0.11 | 0.11 | — | — | — | — | 0.24 | 0.50 |
| MVP5180 | 0.33 | 0.34 | 0.34 | 0.29 | — | — | — | 0.29 | 0.52 |
| ANT70 | 0.35 | 0.35 | 0.34 | 0.31 | 0.10 | — | — | 0.30 | 0.53 |
| ESP1 | 0.36 | 0.35 | 0.34 | 0.32 | 0.10 | 0.05 | 0.51 | 0.29 | 0.53 |
| HIV-2 ROD | 0.52 | 0.48 | 0.50 | 0.50 | 0.49 | 0.51 | — | 0.51 | 0.51 |

[a] The values correspond to the proportion of nucleotides substituted when pairs of *pol* (RT-coding region) sequences were compared applying the Kimura two-parameter model (Kimura, 1980). The genetic distances are expressed as percentage differences.

[b] Subtype U includes unclassified viruses. The MAL isolate has been defined as a recombinant virus of subtypes A and D.

98, 179, and 181, which are involved in resistance to nonnucleoside reverse transcriptase inhibitors. Gly-98 and Glu-179 are found in all group O isolates, instead of Ala and Val, respectively, which occur in group M HIV-1 strains (Fig. 2). ESP1 and ANT70 have Cys at position 181 instead of Tyr as found in MVP5180 and group M HIV-1 isolates. Despite the significant differences observed between group O and group M HIV-1 RT sequences, they

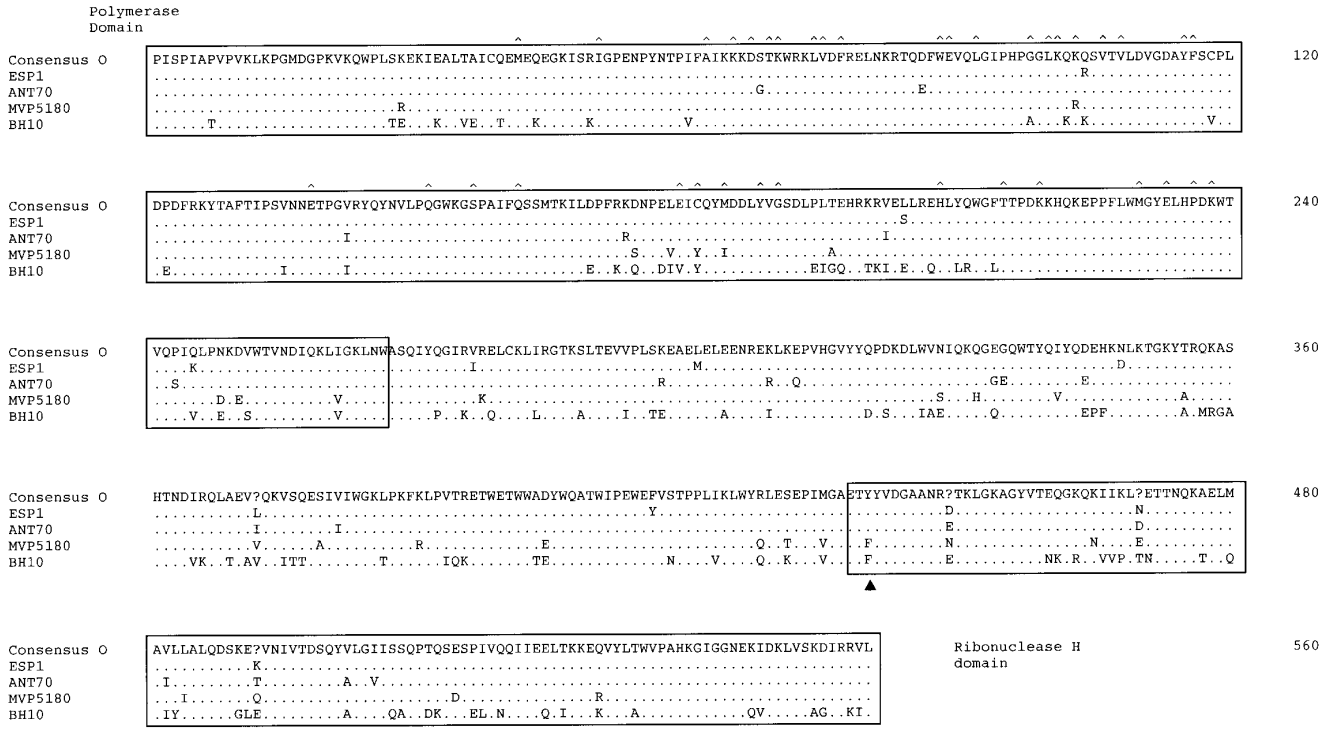


FIG. 2. Alignment of amino acid sequences of RT of HIV-1 group O isolates. The consensus sequence of the group O HIV-1 RT was obtained from the sequences of the Spanish isolate ESP1 described in this paper, ANT70 (Vanden Haesevelde *et al.,* 1994) and MVP-5180 (Gürtler *et al.,* 1994). The sequence of a group M HIV-1 isolate (BH10) is shown for comparison. The single-letter amino acid code is used. Dots represent identical residues as compared with the group O HIV-1 consensus sequence. Residues involved in resistance to RT inhibitors (Domingo *et al.,* 1997) are marked with ^. The polymerase and RNase H domains (Jacobo-Molina and Arnold, 1991) are boxed. A black triangle is used to indicate the processing site between p51 and the RNase H domain (Chattopadhyay *et al.,* 1992).

are more closely related to each other than to the RT of HIV-2. The percentage of amino acid changes observed in the sequence derived from the RT-coding region of the HIV-$2_{ROD}$ (Guyader *et al.,* 1987) isolate compared with the RTs of group O strains is around 38%.

### Cloning, expression, and purification of group O HIV-1 RT

For the cloning of p66 and p51, it was assumed that the amino- and carboxyl-terminal ends of both subunits were identical to those reported for the RT of HIV-1 subtype B (DiMarzo Veronese *et al.,* 1986; Chattopadhyay *et al.,* 1992). The amino acid sequence around the maturation site at the amino-terminal end of the RT was conserved in all group O viruses, as well as in BH10 (Fig. 2). At the carboxyl-terminal end of p66 and at the p51/RNase H cleavage sites, we observed three conserved substitutions: Lys-558 → Arg, Ile-559 → Val, and Phe-440 → Tyr. These amino acid changes are not expected to have a large effect in processing by the viral protease (Poorman *et al.,* 1991; Chou, 1993). The region coding for the 66-kDa subunit of the ESP1 isolate was amplified by nested PCR using proviral DNA of the infected individual as template. The resulting DNA was then cloned into the pUC-derived plasmid pRT6, using the restriction sites *Nco*I and *Eco*RI. The obtained construct was used for expression of p66. The nucleotide sequence of the insert was identical to the average sequence derived from the PCR product and used in the comparisons and phylogenetic analysis described above. The 51-kDa subunit was obtained after cloning with appropriate primers (Table 2 and Materials and Methods), using the p66 construct as template. The nucleotide sequence of the insert was again identical to that found in the template DNA. Purification of the group O HIV-1 RT was done after independent expression of its subunits. The 51-kDa subunit included six consecutive histidines at its amino terminal extension, to facilitate its purification by metal chelate affinity chromatography. The 66-kDa subunit was constitutively expressed by the bacterial clones, but expression of p51 was induced with isopropyl $\beta$-D-thiogalactoside. Several *E. coli* strains were tested for expression of both subunits. Although expression levels of p66 and p51 were similar in DH5$\alpha$ and in several TOPP strains, the highest purity of the purified heterodimeric RT was obtained with TOPP1 for p66 and DH5$\alpha$ for p51. In this case, enzymes were at least 90% pure, as judged by SDS–PAGE. The overall yield of the process was ~0.05–0.1 mg of protein per liter of bacterial culture.

### Kinetic properties and inhibitor sensitivity of recombinant group O HIV-1 RT

The specific DNA polymerase activity of the purified group O HIV-1 RT was similar to that obtained with purified RT of the BH10 isolate (data not shown; Martín-Hernández *et al.,* 1996). The RNase H-specific activity of the RT of the ESP1 isolate was 298.0 ± 63.0 units/mg. This value was only slightly lower than the one reported for the enzyme of the BH10 isolate (Martín-Hernández *et al.,* 1997). Kinetic parameters for DNA polymerase activity were measured with poly(rA)·oligo$(dT)_{20}$ and dTTP and are shown in Table 3. The values for dTTP incorporation ($k_{cat}$ and $K_m$) were similar for the group O and the group M enzymes, although the affinity for the RNA–DNA complex was somewhat higher in the case of the group O HIV-1 RT.

We tested the inhibitor sensitivity of the group O recombinant RT and compared it with the RT of the BH10 isolate. The nucleotide triphosphate analogues ddTTP and AZTTP inhibited both enzymes in a competitive manner. Although the group O RT appears to be slightly more sensitive to those inhibitors, differences in their inhibition constants were relatively minor (Table 4). Furthermore, the differences between the corresponding $K_i/K_m$ ratios for both enzymes and inhibitors were almost indistinguishable. In contrast, large differences were observed with nonnucleoside RT inhibitors (Table 5). Depending on the homopolymeric template-primer used in our assay conditions, the $IC_{50}$ values for nevirapine and loviride were at least 24 to 1250 times higher with the group O enzyme than with the group M RT.

## DISCUSSION

The genetic distance between the RT-coding region of the HIV-1 group O isolate (ESP1) and the previously characterized ANT70 (Vanden Haesevelde *et al.,* 1994) is about 5%. Their derived amino acid sequences differed in 27 residues. The comparison of the primary structures of HIV-1 RTs of ESP1 and BH10 revealed a 79% amino acid sequence identity between both enzymes. Forty amino acid changes are found in the polymerase domain, but only 16 are conservative substitutions. Site-directed mutagenesis studies using a subtype B clone of HIV-1 RT very similar to BH10, revealed that several of the substitutions leading to amino acids found in the group O enzyme (Glu-169 → Asp, Lys-172 → Arg, Asp-177 → Glu, Ile-178 → Leu, Ile-195 → Leu, Gln-197 → Glu, Thr-200 → Lys, and Ile-202 → Val) did not have a major effect on the DNA polymerase specific activity of the RT (Chao *et al.,* 1995).

Enzymological characterization of the group O HIV-1 RT was carried out after expression and purification of the enzyme derived from the ESP1 isolate. The results indicate that group O and group M enzymes are very similar in terms of DNA polymerization kinetics, and sensitivity to nucleotide analogue inhibitors of RT. For example, the $K_i$ for ddTTP and AZTTP ranged from 2.9 to 14.0 n*M* for both enzymes, with differences of less than threefold for the same inhibitor. These values are similar to those reported for the group M subtype B strain, HXB2 (Martin *et al.,* 1993; Gu *et al.,* 1994). The largest differ-

TABLE 3

Kinetic Parameters for dTTP Incorporation and Estimated $K_d$ Values for Poly(rA)·oligo(dT)$_{20}$ of HIV-1 Group O and Group M Reverse Transcriptases[a]

| Enzymes | $k_{cat}$ ($s^{-1}$) | $K_m$ ($\mu M$) | $k_{cat}/K_m$ ($mM^{-1} \cdot s^{-1}$) | $K_d$ (n$M$) |
|---|---|---|---|---|
| Group O (ESP1) | 0.29 ± 0.10 | 4.0 ± 1.6 | 75.2 ± 16.0 | 1.4 ± 0.2 |
| Group M (BH10)[b] | 0.47 ± 0.16 | 6.7 ± 1.7 | 70.1 ± 10.8 | 7.3 ± 1.8 |

[a] Poly(rA)·oligo(dT)$_{20}$ was used as substrate. The template/primer nucleotide ratio was 10:1 (approximate molar ratio 1:2.5). Data shown are the mean values ± standard deviation, obtained from a nonlinear least squares fit of the kinetics data to the Michaelis–Menten equation. Each of the experiments was performed independently at least three times. The $k_{cat}$ values are given assuming that 50% of the enzyme was active as determined by active site titration (Martin-Hernández *et al.,* 1996).

[b] Reported data for this enzyme were taken from Martín-Hernández *et al.* (1996).

ences between the RT of HIV-1 group M and group O were observed with regard to inhibition by nonnucleoside RT inhibitors such as nevirapine or loviride. The $IC_{50}$ for loviride was in the micromolar range for all tested substrates using the RT of the BH10 isolate, in agreement with the data of Pauwels *et al.* (1993) using an enzyme of a closely related strain of HIV-1. Similar results were obtained with nevirapine. Its $IC_{50}$ (0.16 $\mu M$), determined in the presence of poly(rC)·oligo(dG)$_{12-18}$ and dGTP, using the recombinant RT of the BH10 isolate, falls within the same range of other reported values for the same substrates and closely related RTs (Merluzzi *et al.,* 1990; Byrnes *et al.,* 1993; Ahgren *et al.,* 1995; Zhang *et al.,* 1995). On the other hand, nevirapine and loviride had no effect on the RT of HIV-1 group O ($IC_{50} > 200$ $\mu M$). These results are consistent with previous observations on the phenotypic resistance of HIV-1 group O isolates grown in the presence of nonnucleoside RT inhibitors (Descamps *et al.,* 1995). Three residues of HIV-1 group O RT (Cys-181, Glu-179, and Gly-98) are also found in group M (subtype B) strains resistant to nonnucleoside RT inhibitors (reviewed in Mellors *et al.,* 1996; and Domingo *et al.,* 1997). The substitution of Tyr-181 by Cys has been shown to confer high-level resistance to loviride ($\alpha$-anilinophenylacetamide derivative R 89439), nevirapine, and practically all tested nonnucleoside RT inhibitors (Pauwels *et al.,* 1993; Richman *et al.,* 1994; Mellors *et al.,* 1996, Domingo *et al.,* 1997). This substitution has not been found in other RTs of HIV-1, HIV-2, or SIV, but it is present in HIV-1 group O isolates ESP1 and ANT70. Analysis of the surface area of the RT in contact with nevirapine or the $\alpha$-anilinophenylacetamide derivative R 95845 (a loviride enantiomer) based on their reported crystal structures (Kohlstaedt *et al.,* 1992; Ding *et al.,* 1995), revealed that Tyr-181 makes close contact with both inhibitors (Erickson and Burt, 1996).

Two additional residues found in group M HIV-1 RT (Val-179 and Lys-102) which are located within van der Waals contact distance with nevirapine and $\alpha$-anilinophenylacetamide derivative R 95845 are also substituted in the RT of the ESP1 isolate. The contribution of Lys-102 to the contact surface area is very small, and to our knowledge, amino acid replacements at this position have not been associated with the acquisition of resistance to nonnucleoside RT inhibitors. However, the contribution of Val-179 is significant, particularly in complexes between RT and the $\alpha$-anilinophenylacetamide derivative R 95845 (Ding *et al.,* 1995; Erickson and Burt, 1996). Group O HIV-1 isolates including ESP1 have Glu at position 179, instead of Val as occurs in group M strains. In the RTs of HIV-2 and SIV, Ile, Leu, Thr, Asp, and Glu are also found at this position, while in group M HIV-1, the Val-179 → Glu substitution has been reported to confer some resistance to pyridinone L-697,661, and TIBO R82913 (Byrnes *et al.,* 1993). Site-directed mutagenesis experiments revealed that in these viruses, the similar substitution of Val-179 by Asp renders an enzyme with a wild-type DNA polymerase activity (Boyer *et al.,* 1993). Furthermore, this amino acid change confers resistance to several groups of nonnucleoside inhibitors of RT (e.g., nevirapine, TIBO compounds, delavirdine, pyridinones, HEPT derivatives, quinoxaline derivatives, and several thiocarboxyanilides) (Mellors *et al.,* 1996, and references therein).

In subtype B HIV-1 strains, the substitution of Ala-98 by Gly has been shown to confer low-level resistance to pyridinones, nevirapine, TIBO derivative R82913, and several thiocarboxyanilides (Byrnes *et al.,* 1993; Buckheit *et al.,* 1995). Ala-98 is found in all group M strains of HIV-

TABLE 4

Inhibition of HIV-1 Group O and Group M Reverse Transcriptases by Dideoxynucleotide Triphosphates[a]

| Enzymes | $K_i$ (n$M$) | |
|---|---|---|
| | ddTTP | AZTTP |
| Group O (ESP1) | 5.9 ± 0.4 | 2.9 ± 1.6 |
| Group M (BH10) | 14.0 ± 2.1 | 6.3 ± 3.2 |

[a] Poly(rA)·oligo(dT)$_{20}$ was used as substrate. The template/primer nucleotide ratio was 10:1 (approximate molar ratio 1:2.5). Data shown are the mean values ± standard deviation of at least three independent experiments.

TABLE 5

Inhibition of HIV-1 Group O and Group M Reverse Transcriptases by Nevirapine and Loviride[a]

| Enzymes | Template/primer | $IC_{50}$ ($\mu M$) | |
|---|---|---|---|
| | | Nevirapine | Loviride |
| Group O (ESP1) | poly(rA)·oligo$(dT)_{20}$ | >200 | >200 |
| | poly(rA)·oligo$(dT)_{12-18}$ | >200 | >200 |
| | poly(rC)·oligo$(dG)_{12-18}$ | >200 | >200 |
| | poly(dC)·oligo$(dG)_{12-18}$ | >200 | >200 |
| Group M (BH10) | poly(rA)·oligo$(dT)_{20}$ | 2.4 ± 0.4 | 4.7 ± 0.7 |
| | poly(rA)·oligo$(dT)_{12-18}$ | 1.2 ± 0.1 | 8.2 ± 1.4 |
| | poly(rC)·oligo$(dG)_{12-18}$ | 0.16 ± 0.02 | 0.5 ± 0.2 |
| | poly(dC)·oligo$(dG)_{12-18}$ | 1.2 ± 0.2 | 6.0 ± 1.2 |

[a] The template/primer nucleotide ratio was 10:1 when poly(rA)·oligo$(dT)_{20}$ was used as substrate, 1:1 for poly(rA)·oligo$(dT)_{12-18}$ and poly(rC)·oligo$(dG)_{12-18}$, and 2:1 for poly(dC)·oligo$(dG)_{12-18}$. Tritium-labeled dTTP and dGTP were used as substrates at a 2 $\mu$M concentration. Reported data (mean value ± standard deviation) were obtained using duplicate determinations with saturating levels of template-primer, at seven or eight different inhibitor concentrations. Each experiment was done at least twice for each enzyme and template-primer.

1 as well as in most of HIV-2 and SIV isolates. However, Gly-98 occurs in all the RT sequences of group O viruses and has been observed in an SIV isolate from African Sykes' monkeys (Hirsch *et al.,* 1993). In summary, the observed amino acid substitutions at the nonnucleoside RT inhibitor binding site of group O HIV-1 RT can explain the differences in inhibitor sensitivity between group M and group O RTs.

HIV-2 isolates are also resistant to nonnucleoside RT inhibitors, an effect that appears to be mediated by the presence of Ile and Leu at positions 181 and 188 of the RT sequence, respectively (Loya *et al.,* 1994). However, the sequence and enzymological properties of group O HIV-1 RT suggest a closer relationship of this enzyme with the group M enzyme than with the HIV-2 RT. HIV-1 group M (subtype B strains) and HIV-2 RTs have similar polymerase activity, affinity for homopolymeric RNA–DNA template-primers, and sensitivity to ddTTP and AZTTP (Hizi *et al.,* 1991), but differ in their specific RNase H activity which is 10 times lower in HIV-2 RT when measured in the presence of $Mg^{2+}$, using [$^3$H]-poly(rA)·poly(dT) as substrate (Hizi *et al.,* 1991; Fan *et al.,* 1996). Our results showed that in the case of group O HIV-1 RT, the RNase H specific activity was similar to that shown by the RT of the BH10 isolate. Hizi *et al.* (1991) have also reported differences between both enzymes in their affinity for dTTP, which is apparently lower for HIV-2 RT. However, only minor differences were detected by Fan *et al.* (1996) and Chattopadhyay *et al.* (1992) when HIV-1 and HIV-2 RTs were compared.

Although most of the reported cases of HIV-1 group O infection have been identified in persons living or coming from Cameroon and neighboring countries (Mauclère *et al.,* 1997; Peeters *et al.,* 1997), evidence of spread of group O viruses outside Africa has been recently documented (Charneau *et al.,* 1994; Hampl *et al.,* 1995; Gould *et al.,* 1996; Soriano *et al.,* 1996). Successful antiviral drugs must be able to cope with an immense diversity of viral genotypes which may prevail in different human populations. It is also possible that different strains acquire resistance to inhibitors through different patterns of amino acid substitutions. From this point of view, the purified recombinant group O HIV-1 RT is expected to be a valuable tool to address those questions.

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